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Gonadotropin-Releasing Hormone Binding to Rat Anterior Pituitary Membrane Homogenates

Comparison of Antagonists and Agonists Using Radiolabeled Antagonist and Agonist

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SUMMARY

The gonadotropin-releasing hormone (GnRH) antagonist (acetyl-Δ^3-Pro^1, *p*-fluoro-D-Phe^2, D-Trp^3, D-Lys^6)-GnRH (An) has been radioiodinated and its binding to rat anterior pituitary membrane homogenates is shown to be saturable, specific, and of high affinity ($K_a = 6 \times 10^9$ M^{-1}). The binding of this antagonist is compared with that of an agonist, (D-Ala^6,N^α-methyl-Leu^7,Pro^9-*N*-ethyl)-GnRH (Ag), with similar apparent affinity ($K_a = 3 \times 10^9$ M^{-1}) at equilibrium. The association rates for the two compounds are similar (13×10^7 min^{-1} M^{-1}), as are the dissociation rates (0.015–0.018 min^{-1}). Using a computerized nonlinear regression analysis, it is shown that the number of high-affinity sites to which the antagonist binds is slightly greater than the number to which the agonist binds. This small difference may be merely a result of nonspecific hydrophobic interactions between the antagonist and the crude membrane homogenates. More important, the relative potencies of representative GnRH analogues determined from competitive binding assays using the labeled antagonist are not significantly different from those determined from assays using the labeled agonist. The relative potencies of Ag and An measured using either radioligand are unaffected by temperature, pH, Na^+, Ca^{2+}, or guanyl nucleotides.

INTRODUCTION

GnRH[2] (*p*Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) acts on the pituitary to stimulate secretion of gonadotropins (1) and on the ovary to inhibit follicle-stimulating hormone-induced steroidogenesis (2). Work in several laboratories (3–5) has led to the development of highly potent antagonists which, *in vivo,* inhibit the actions of exogenous and (in some cases) endogenous GnRH on its target organs and, *in vitro,* reduce the amount of luteinizing hormone secreted by cultured anterior pituitary cells in response to GnRH.

The initial action of GnRH on its target tissues is presumably to bind to specific receptors on the plasma membrane. In order to probe the mechanism of action of GnRH and the factors that distinguish agonists from antagonists, it is desirable to study and compare the binding of the two classes of ligands. Most previous radioreceptor studies describing high-affinity binding of GnRH analogues to cultured rat anterior pituitary cells (6) and to rat and bovine anterior pituitary membrane homogenates (7–12) have employed either radiolabeled GnRH or GnRH agonists (6–12). Recently, Meidan and Koch (13) have carried out studies on the binding of the antagonist (D-*p*Glu^1,D-Phe^2,D-Trp^{3,6})-GnRH to dispersed rat anterior pituitary cells, and Hazum (14) has reported binding of the same antagonist to membrane homogenates.

Previously, we have found that iodination of the antagonist (Ac-Δ^3-Pro^1,*p*F-D-Phe^2,D-Trp^{3,6})-GnRH produced a radioligand that had so much nonspecific and low-affinity binding that it was virtually useless for radioreceptor studies.[3] We reasoned that reducing the hydrophobic character of the molecule by substituting a Lys at position 6 might decrease the nonspecific binding while maintaining the potency of the antagonist. It was found that (Ac-Δ^3-Pro^1,*p*F-D-Phe^2,D-Trp^3,D-Lys^6)-GnRH (An) had high affinity for the pituitary in a radioreceptor

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[2] The abbreviations used are: GnRH, gonadotropin-releasing hormone; An, antagonist [(acetyl-Δ^3-Pro^1,*p*-fluoro-D-Phe^2,D-Trp^3-D-Lys^6)-GnRH]; Ag, agonist [(D-Ala^6,N^α-methyl-Leu^7,Pro^9-*N*-ethyl)-GnRH]; HPLC, high-pressure liquid chromatography; BSA, bovine serum albumin, crystallized (Pentex).

[3] M. H. Perrin, Y. Haas, J. E. Rivier, and W. W. Vale, unpublished data.

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assay and was an effective antagonist both *in vitro* and *in vivo*.

We wish to report here the radioiodination of this antagonist and its potential usefulness as a radioligand for radioreceptor studies of the pituitary. The binding of this antagonist to rat anterior pituitary membrane homogenates is characterized and compared with that of the agonist, (D-Ala^6,N^{α}-MeLeu^7,Pro^9-NEt)-GnRH (Ag). Furthermore, the relative potencies of representative GnRH analogues determined from assays using both radioligands are given and are shown to be independent of which radioligand is used.

MATERIALS AND METHODS

Preparation of tissues. Following decapitation of male Sprague-Dawley rats, anterior pituitaries were removed and either frozen on dry ice and stored at −20° for short periods of time, or kept on ice for immediate membrane preparation. After homogenization by hand (10 times, glass-Teflon homogenizer) in 10 volumes 0.32 M sucrose on ice, the mixture was centrifuged at 600 × *g* for 5 min and the resulting supernatant was centrifuged at 48,000 × *g* for 20 min at 4°. The resulting pellet, P_2, was suspended in 0.32 M sucrose to a concentration of 10–20 mg of protein per milliliter. The final suspension was frozen in liquid N_2 and stored at −20°.

Peptide synthesis. Peptides were synthesized by solid phase according to well-established procedures (15). After purification, the peptides were characterized by amino acid analysis and were found to have a purity greater than 90% by HPLC and thin-layer chromatography in several solvent systems.

Radioiodination. The peptide (Ag or An) (10 μl, 0.1 mg/ml in 5 mM acetic acid) was added to 0.5 M phosphate buffer (pH 7.6) (20 μl) and 1 mCi of ^{125}I-Na [carrier-free, low pH; New England Nuclear Corporation (Boston, Mass.)]. To this solution was added chloramine T [20 μl, 0.04 mg/ml in 0.05 M phosphate buffer (pH 7.6)]. The solution was gently vortexed for 30 sec and the reaction was then terminated by adding $Na_2S_2O_5$ [100 μl, 1.2 mg/ml in 0.05 M phosphate buffer (pH 7.6)/5% BSA] and vortexing for 30 sec. The solution was diluted to 2 ml with 5 mM NH_4OAc (pH 4.5) and applied to a carboxymethylcellulose (CM-52) column (volume 1 ml) which had been equilibrated with 5 mM NH_4OAc (pH 4.5). The column was eluted using a peristaltic pump at a constant rate of 100 μl/min first with 5 mM NH_4OAc (pH 4.5) (26 ml) and then with 125 mM NH_4OAc (pH 4.5). Fractions were collected every 5 min in borosilicate glass tubes. The fractions which were assumed to contain the iodinated peptide were pooled, lyophilized, and further purified by HPLC. An alternative purification eliminates the use of CM-52 and uses instead a SEP-PAK C cartridge (Waters Associates, Inc., Milford, Mass.) to which the iodination mixture is applied following dilution with 5 mM NH_4OAc. The cartridge is eluted successively with 5 ml of water, 5 ml of HOAc (0.5 N), and 5 ml of 75% CH_3CN/25% HOAc (0.5 N). The iodinated peptide, which is contained in the last elution buffer, is then further purified by HPLC. The radiolabeled agonist is applied to an Altex Model 332 HPLC apparatus and eluted isocratically with 73.6% triethylammonium formate (pH 3.0) and 26.4% acetonitrile as detailed by Perrin *et al.* (7). The iodinated antagonist is eluted using triethylammonium formate and an acetonitrile gradient (21%–36% in 25 min) at a flow rate of 1.5 ml/min on a Waters C_{18} column (0.39 × 30 cm). The solution containing the iodinated peptide to which is added BSA (final concentration 0.2%) is lyophilized, resuspended 10 mM acetic acid (10^6 cpm/μl), and stored in a cold room. Radiolabeled agonist ([^{125}I]Ag) and antagonist ([^{125}I]An) were shown by HPLC to coelute with the corresponding iodinated peptides (m-I-Ag and m-I-An) synthesized *de novo*.

Binding assay. The peptides, initially dissolved (1 mg/ml) in acid (0.01–1 N HOAc) and the radioligand, all diluted in assay buffer (0.2% or 1% BSA/10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6) were added to duplicate or triplicate borosilicate glass tubes on ice. Binding was initiated by the addition of the membrane homogenate (20–30 μg of P_2), and the incubation (in a total volume of 100 μl) was continued for 120 min. The reaction was terminated by the addition of 1–2 ml of ice-cold saline, and the mixture was poured immediately onto Whatman GF/C filters (wetted in a 1% BSA assay buffer) and filtered under gentle suction. The incubation tube was washed once with 1–2 ml of ice-cold saline and the filters were washed eight times with approximately 1 ml of saline per wash. The total washing procedure took about 10 sec. The filters were then counted in a Searle γ-counter at 85% efficiency. Each assay contained duplicate tubes without membranes as a measure of background binding to the filter paper. Protein concentrations were determined with a Bio-Rad protein kit (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

The availability of the labeled antagonist and agonist along with their (unlabeled) iodinated analogues (whose syntheses were necessary for the correct mathematical analysis) allows a direct comparison of the membrane binding of these two compounds. Analysis of the competitive displacements of the radioligands by each of the (unlabeled) iodinated analogues permits the determination and comparison of the total number of binding sites as well as of the affinities. In Fig. 1A and B we show the results (expressed as B/T) of a homologous displacement experiment performed simultaneously with both the antagonist (m-I-An) and the agonist (m-I-Ag), using one membrane preparation. The experimental data are represented by the distinct points and are compared with the calculated curve representing the best computerized fitting of the data (see below). In the *insets* to Fig. 1 we display the experimental data in Scatchard representations in which the experimental points are shown together with the best-fitted lines whose slopes and intercepts represent the calculated affinities and total binding site concentrations, respectively. It is clear from the Scatchard plot that there is a much greater "low-affinity" binding (or of binding to more than one site) for the antagonist than for the agonist.

Because there is such a large component of low-affinity binding, it would be of dubious validity to use a simple least-squares analysis of the Scatchard plot. Instead, the generalized, nonlinear regression analysis (known in a computerized version called LIGAND) developed specif-

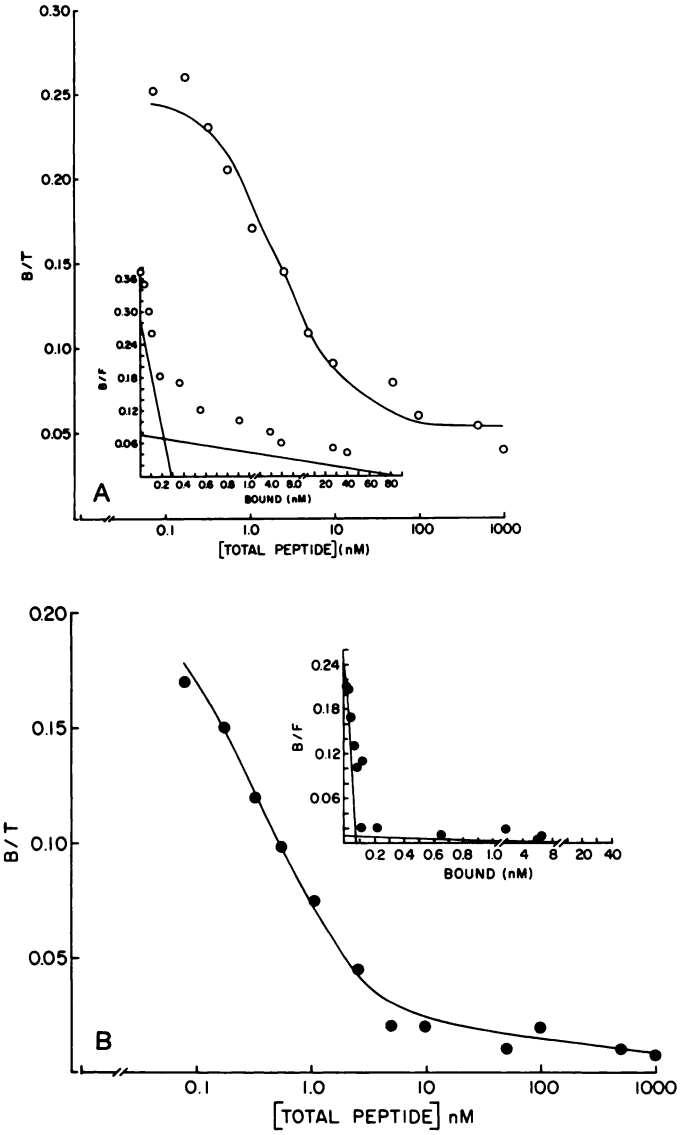

FIG. 1. *Displacement of [^{125}I]An by m-I-An (A) and of [^{125}I]Ag by m-I-Ag (B)*

Approximately 18,000 cpm of [^{125}I]An or 27,000 cpm of [^{125}I]Ag were incubated with 30 μg of P_2 and increasing concentrations of either m-I-An or m-I-Ag (respectively) for 2 hr on ice. Background binding to filters (~3% for both agonist and antagonist) (in the absence of P_2) was subtracted from all counts. The calculated curve from the LIGAND program (*solid line*) is compared with the experimental data (*points*). *Insets*, Scatchard representation of the data. The *points* are experimental, and the straight lines represent the best nonlinear regression values for the affinities and concentration of high- and low-affinity sites.

ically for radioreceptor studies is the method of choice (16). The strength of this analysis lies in its use of the exact mathematical model that characterizes radioreceptor systems. The parameters obtained from the LIGAND analysis of the data in Fig. 1A and B are given in Table 1A, where we compare the one-site model with the two-site model and give a statistical estimate (F-test) of the necessity for including a second site in the analysis. In agreement with the qualitative conclusion drawn from the Scatchard plot in Fig. 1B, the inclusion of a second site for the agonist does not really change the parameters or significantly improve the fit, but consideration of the second site is crucial for the antagonist. Furthermore, for the antagonist there appears to be a small residual binding even at 1000 nM, so there may be other classes of sites of even lower affinity.

The usual procedure for examining the effect of the other, lower, affinity sites is to subtract the "nonspecific" binding, which is usually defined as the binding in the presence of excess (e.g., 1000 nM) unlabeled peptide. This procedure may be applied here, and the data may again be analyzed by the LIGAND program. The computerized fitting after subtracting the counts bound in the presence of 1000 nM peptide is given in Table 1B. Removing the nonspecific binding removed the effect of the low-affinity sites in the case of the agonist, but there still was a statistically significant contribution to the high-affinity binding parameters for the antagonist. It is statistically advantageous to retain the "nonspecific" binding as a parameter and allow the program to include it in the fitting procedure (16). This is the procedure that was followed for the analysis of all other experiments. The average value (from five determinations) for the high-affinity K_a for the iodinated agonist was 4.4 (3.9–4.9) $\times$ 10^9 M^{-1} and for the iodinated antagonist (from six determinations) it was 2.6 (2.0–3.5) $\times$ 10^9 M^{-1}.

As shown in Table 1A, the number of both high- and low-affinity sites for the antagonist was only slightly greater than that for the agonist. From four determinations using different membrane preparations, the average ratio of antagonist to agonist binding site concentration was found to be 2.3 (1.4–2.7). Determination of the total number of binding sites, especially the low-affinity sites, is difficult because the calculation is sensitive to the fine details of the displacement curves in the high-dose region, where the binding is low. It is possible that the small difference in sites reflects variabilities in membrane preparations leading to variable nonspecific low-affinity interactions, which were not completely accounted for by the LIGAND program.

In Fig. 2 we show the competitive displacements of [^{125}I]An and [^{125}I]Ag by both Ag and An. Both experiments were performed in the same assay using the same membrane preparation. Whereas both radioligands appeared similar in the high-affinity regions, there was a small fraction of labeled antagonist that was not displaced by high doses of agonist. However, the antagonist did displace all of the bound [^{125}I]Ag. It may be the case that the antagonist bound to a set of sites which were inaccessible to the agonist. To test, by means of the LIGAND program, the model in which there are different low-affinity sites for the agonist and antagonist, we may combine these two experiments with the other experiments in which each radioligand was displaced by its corresponding cold iodinated analogue. In Table 2 we list the computerized analyses of all four experiments. The model in which the agonist and antagonist bound to different low-affinity sites (but the same high-affinity site) gives a much better fit [F = 101 (p = 0)] than the model in which both compounds bound to the same low-affinity site. Thus, at least for this membrane preparation, it may be true that the agonist and antagonist bind to different low-affinity sites.

TABLE 1

Nonlinear regression parameter estimates for the data in Fig. 1

	K_{a_1}	R_1	K_{a_2}	R_2	N	F
	nM^{-1}	nM	nM^{-1}	nM		
Part A[a]						
m-I-An						
One-site model	0.66 ± 0.22	0.43 ± 0.11	—	—	0.06	
Two-site model	1.1 ± 0.2	0.26 ± 0.04	0.00084 ± 0.00018	88 ± 15	0	43 ($p = 0$)
m-I-Ag						
One-site model	2.5 ± 1.1	0.093 ± 0.024	—	—	0.01	
Two-site model	3.2 ± 1.5	0.075 ± 0.019	0.0011 ± 0.0006	14 ± 6	0	6.5 ($p = 0.03$)
Part B[b]						
m-I-An						
One-site model	0.55 ± 0.21	0.44 ± 0.13	—	—	0.02	
Two-site model	1.5 ± 0.8	0.18 ± 0.8	0.016 ± 0.017	2.5 ± 1.8	0.008	9.4 ($p = 0.01$)
m-I-Ag						
One-site model	3.1 ± 1.4	0.076 ± 0.017	—	—	0.008	
Two-site model	No meaningful fitting					

[a] These data were fitted without subtracting the counts bound in the presence of 1000 nM cold peptide. N, nonspecific binding, was treated as a variable parameter (16).

[b] The counts bound in the presence of 1000 nM cold peptide were subtracted from all of the data before computer analysis. N was again treated as a variable parameter (16).

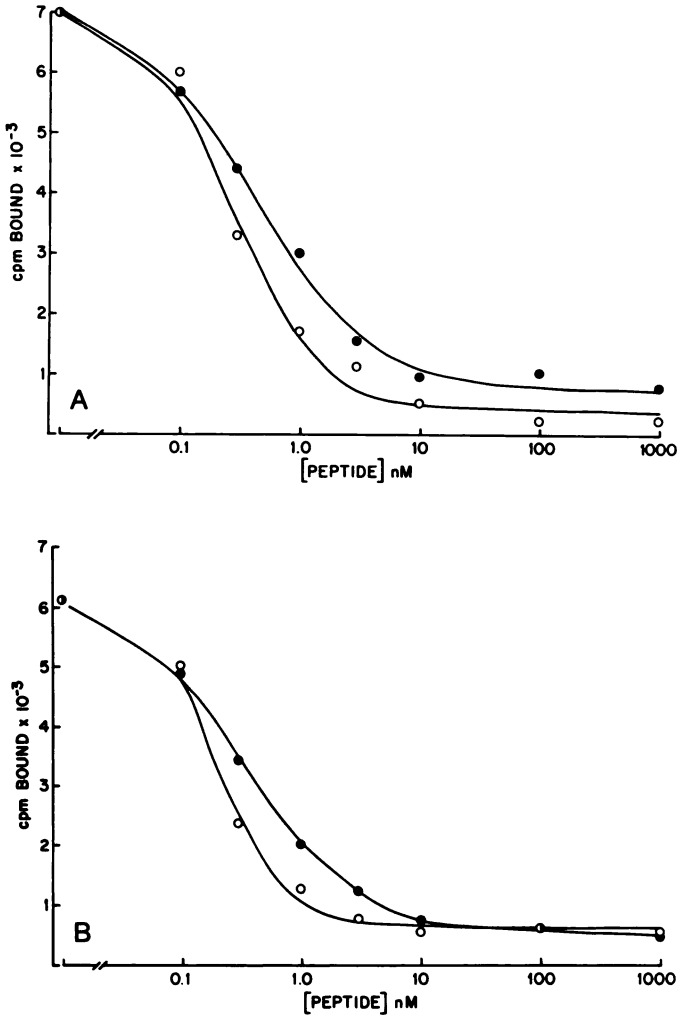

FIG. 2. *Competitive displacement of [^{125}I]An and [^{125}I]Ag by Ag and An*

Approximately 25,000 cpm of [^{125}I]An (A) or 23,000 cpm of [^{125}I]Ag (B) were incubated with 30 μg of P_2 for 2 hr on ice and increasing concentrations of An (○) and Ag (●). The reaction was terminated and the mixture was filtered as described under Materials and Methods. Background binding to the filter paper (10% for [^{125}I]An, 2% for [^{125}I]Ag) was subtracted from all counts per minute. The *points* are experimental and the *curves* are the best fitting according to ALLFIT (17).

TABLE 2

Comparison of the model in which the agonist and antagonist share the same low-affinity sites with the model in which they share different low-affinity sites

Model	K_{a_1}	K_{a_2}	K_{a_3}	Total mean square	F
	nM^{-1}	nM^{-1}	nM^{-1}		
Same low-affinity sites					
m-I-An	1.2 ± 0.5	0.005 ± 0.009	—		
m-I-Ag	2.6 ± 1.1	0		1282	—
Different low-affinity sites					
m-I-An	2.4 ± 0.4	0	0.003 ± 0.003		
m-I-Ag	3.5 ± 0.6	0.3 ± 0.1	0	99	101 ($p = 0$)

In Fig. 3 we show the rates of association (A) and dissociation (B) for the two radioligands. Both compounds displayed similar kinetics. A least-squares fitting of the data yielded a value of $(13 \pm 2) \times 10^7$ min^{-1} M^{-1} for the second-order association rate constant for the antagonist and $(13 \pm 0.7) \times 10^7$ min^{-1} M^{-1} for the agonist. If we assume simple first-order kinetics for the dissociation, a least-squares fitting of the data yields a value of (0.015 ± 0.002) min^{-1} for the antagonist and (0.018 ± 0.002) min^{-1} for the agonist. By taking the ratio of these rate constants we obtain a value of 8.6×10^9 M^{-1} for the K_a of the antagonist and 2.2×10^9 M^{-1} for the K_a of the agonist. These values compare favorably with the average for the high-affinity K_a values obtained from equilibrium displacement data.

In Fig. 4 we show the effect of increasing membrane concentration, and in Fig. 5 the effect of pH on the

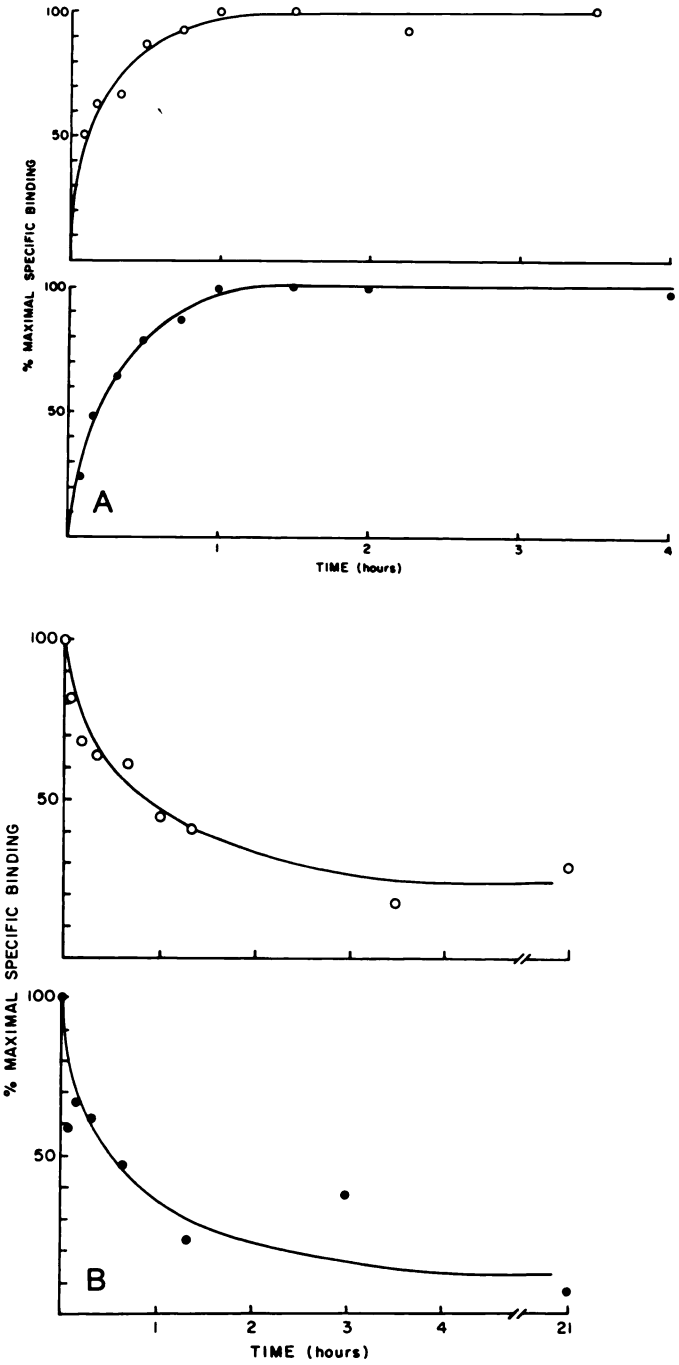

FIG. 3. *Rates of association and dissociation for [^{125}I]An and [^{125}I]Ag*

A. Approximately 46,000 cpm of [^{125}I]An (○) were incubated with 30 μg of P_2, and approximately 40,000 cpm of [^{125}I]Ag (●) were incubated with 20 μg of P_2. The reaction was stopped at the indicated times by the addition of 1 ml of cold saline and promptly filtered as described under Materials and Methods. Specific binding is defined as counts per minute bound in the absence of cold peptide minus counts per minute bound in the presence of 1000 nM cold peptide. The normalized specific binding is plotted.

B. Approximately 46,000 cpm of [^{125}I]An (○) were incubated with 30 μg of P_2, and 30,000 cpm of [^{125}I]Ag (●) were incubated with 20 μg of P_2. Excess cold buffer (10 times the incubation volume) was added after the reaction had reached equilibrium, and the mixture was filtered at the indicated times after this addition, as described under Materials and Methods. The specific binding (normalized), as defined in A, is plotted.

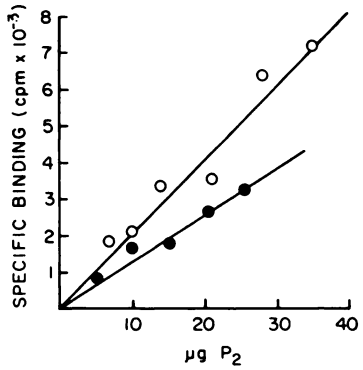

FIG. 4. *Effect of increasing membrane concentration on specific binding of [^{125}I]An and [^{125}I]Ag*

Approximately 30,000 cpm of [^{125}I]An (○) or 25,000 cpm of [^{125}I]Ag (●) were incubated with increasing concentrations of P_2 for 2 hr on ice. The reaction was terminated and the mixture was filtered as described under Materials and Methods. The specific binding, as defined in Fig. 3A, is plotted.

specific binding of [^{125}I]An and [^{125}I]Ag. The agonist and antagonist showed similar behavior. The values of membrane homogenate used for the binding assay (20–30 μg of P_2) were in an appropriate range, and a pH ~ 7.5 was suitable for both radioligands.

The relative potencies of GnRH analogues obtained from competitive displacement assays using the labeled agonist may now be compared with the potencies deter-

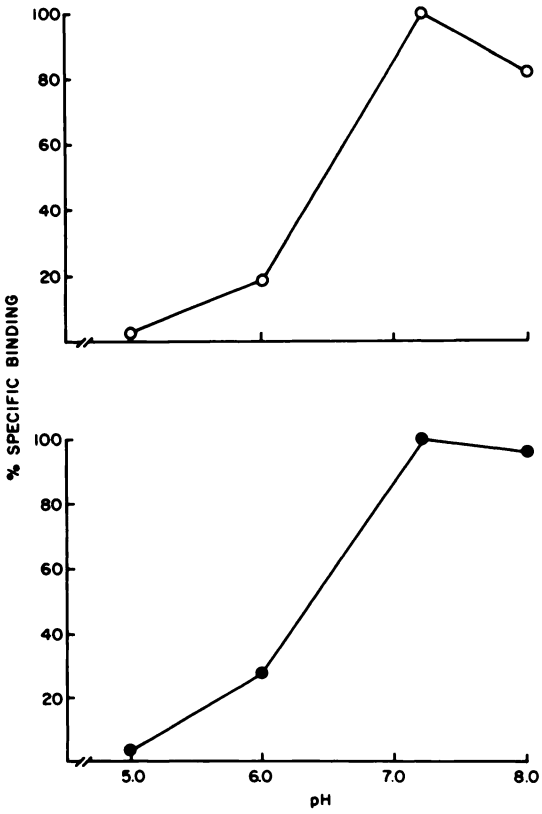

FIG. 5. *Effect of pH on specific binding of [^{125}I]An and [^{125}I]Ag*

[^{125}I]An (○; 60,000–80,000 cpm) or [^{125}I]Ag (●; 40,000–50,000) was incubated with 30 μg of P_2 for 90 min on ice in assay buffer whose pH was adjusted with either KOH or HOAc. The normalized specific binding, as defined in Fig. 3A, is plotted.

TABLE 3

GnRH analogue affinities determined using labeled agonist and labeled antagonist

Compound	[^{125}I]Ag		[^{125}I]An	
	K_a (range)	No. times tested	K_a (range)	No. times tested
	nM^{-1}		nM^{-1}	
(D-Ala^6,N^{α}-MeLeu^7,Pro^9-NEt)-GnRH	3.5 (3.0–4.1)	2	3.1 (1.6–5.8)	4
(Ac-Δ^3-Pro^1,*p*F-D-Phe^2,D-Trp^3,D-Lys^6)-GnRH	5.0 (3.8–6.6)	2	6.2 (4.3–9.0)	2
(m-I-Tyr^5,D-Ala^6,N^{α}-MeLeu^7,Pro^9-NEt)-GnRH	4.4 (3.9–4.9)	5	2.0 (1.1–3.4)	1
(Ac-Δ^3-Pro^1,*p*F-D-Phe^2,D-Trp^3,m-I-Tyr^5,D-Lys^6)-GnRH	2.2 (1.7–2.8)	1	2.6 (2.0–3.5)	6
GnRH	0.17 (0.12–0.20)	3	0.39 (0.23–0.66)	1
(D-Trp^6)-GnRH	5.0 (3.8–6.5)	2	3.9 (2.6–5.9)	2
(D-Trp^6,Pro^9-*N*-NEt)-GnRH	8.8 (6.9–11)	2	6.5 (4.1–10)	2
Thyrotropin-releasing hormone	<0.001	1	<0.001	1
Somatostatin	<0.001	1	<0.001	1
Corticotropin-releasing factor	<0.001	1	<0.001	1

mined from assays using the labeled antagonist. In Table 3 we list the affinities (K_a) for some representative GnRH analogues. These values were calculated from the analogues' relative potencies as determined from assays (using at least six doses for each analogue) in which An or Ag was used as the standard for [^{125}I]An or [^{125}I]Ag, respectively. The K_a values of these standard compounds were in turn calculated from their potencies relative to m-I-An and m-I-Ag, whose affinities were taken as their average values obtained from the LIGAND program. These relative potencies were determined from a computer program that uses a simultaneous analysis of the displacement data (ALLFIT) (17). The final values are taken as a weighted average of the individual experiments. It is clear that the affinities (and therefore the relative potencies) are independent of which radioligand is used.

In other systems for which antagonists exist, differential effects on agonist and antagonist affinities have been found. For example, guanyl nucleotides differentially affect muscarinic (18) and dopaminergic (19) receptor binding. Sodium ions differentially enhance opiate antagonist binding (20), and act synergistically with guanyl nucleotides to decrease the affinity of the cardiac muscarinic cholinergic receptor for agonists (21). For the β-adrenergic receptor in turkey erythrocytes (22) and in S49 lymphoma cells (23), increasing the temperature reduces the affinity of agonists in both systems but does not affect that of antagonists in the erythrocytes and increases the antagonist potency in the lymphoma cells. Using both the labeled antagonist and the labeled agonist, we have been unable to detect any differential effects of guanyl nucleotides, Na^+, Ca^{2+}, or temperature on the relative affinities of the two compounds. Thus, for the GnRH system, the factors that differentiate the binding of agonists from antagonists await elucidation.

DISCUSSION

From the data presented in Fig. 1 and Table 3 we may conclude that the binding of An to rat anterior pituitary membrane homogenates satisfies the criteria of saturability, high affinity, and specificity.

From the computer analysis of the displacement data using m-I-An it was found that the binding to the low-affinity sites exerts a significant effect on the analogue's affinity. In order to determine the total number of sites and the affinity for the antagonist, it was necessary to include a second low-affinity site, and this was true even if the "nonspecific" binding was subtracted. As was discussed by Munson and Rodbard (16), the inclusion of a second site whose existence is determined to be statistically significant (*F*-test) may result in a reduction of the precision of the final parameters, because of the interdependence of the parameters in the more complex model. However, failure to include this second site (even though poorly determined) would result in an underestimate for the affinity and an overestimate for the total number of sites. This effect is striking in the case of the antagonist, where the high-affinity binding is partially "masked" by the large amount of low-affinity binding.

It was shown by Clayton *et al.* (11) that there is a low-affinity site on the pituitary which accounts for approximately 75% of the specific binding of [^{125}I]GnRH. However, the low-affinity binding of [^{125}I]GnRH appears to be of a different character from that found here, because all of the bound [^{125}I]GnRH was displaceable by both GnRH and an unlabeled agonist; furthermore, the labeled agonist was completely displaced by GnRH.

Preliminary experiments (data not shown) with two other labeled antagonists, (Ac-Δ^3-Pro^1,*p*F-D-Phe^2,D-Trp^{3,6}-GnRH and (Ac-Δ^3-Pro^1,*p*Cl-D-Phe^2,D-Trp^{3,6},N^{α}-MeLeu^7)-GnRH have shown that there is a certain fraction of bound radioligand that is not displaceable even by large doses of unlabeled agonists. All of these highly potent antagonists are quite hydrophobic in nature. In contrast, the other reports of the binding of the labeled antagonist (D-*p*Glu^1,D-Phe^2,D-Trp^{3,6})-GnRH (13, 14), which is less hydrophobic and much less potent, is characterized by one high-affinity site and very low nonspecific binding. It is possible that the low-affinity binding of the antagonists described here represents a nonspecific interaction resulting from the hydrophobic nature of the compounds. It is also possible that the slight difference in numbers of binding sites for the agonist and antagonist is a result of these nonspecific hydrophobic interactions that may not have been completely taken into account by the LIGAND program. At this time it would be

difficult to distinguish between such nonspecific interactions and low-affinity sites.

We have shown that association rates and dissociation rates for both compounds are not significantly different and that these rates are consistent with the affinities determined from equilibrium data. From the association data it can be seen that the antagonist-receptor complex is stable for at least 4 hr, as is the case for the agonist. In other experiments using another iodinated antagonist, (Ac-Δ^3-Pro^1,*p*F-D-Phe^2,^{125}I-Tyr^5,D-Trp^{3,6})-GnRH, it was found that the receptor-antagonist complex was stable for up to 20 hr.[3] Furthermore, it was found that the affinities of some representative analogues as determined using the labeled antagonist are not significantly different from their affinities determined with the labeled agonist.

Clayton and Catt (10) studied a series of agonists and antagonists, using a labeled superagonist, and showed that there was a good correlation between binding affinity and biological activity for both types of analogues. Using a labeled antagonist, Meidan and Koch (13) found parallelism of the inhibition curves of an unlabeled agonist and antagonist and determined that the numbers of receptor sites for both compounds were similar, suggesting that both the agonist and the antagonist bind to the same receptor sites.

We have extended these studies by presenting a detailed mathematical analysis of the binding of a potent antagonist, including a simultaneous analysis of both homologous (same labeled and unlabeled ligand) and heterologous (different labeled and unlabeled ligand) experiments. This analysis was made possible by the synthesis of the model nonradioactive iodinated compounds. In addition, we have calculated the absolute affinities of both agonists and antagonists based on displacement assays using both radioligands.

In the report by Meidan and Koch (13) describing the binding of the labeled antagonist (D-*p*Glu^1,D-Phe^2,D-Trp^{3,6})-GnRH to dispersed pituitary cells, the affinity of the antagonist was 0.76×10^9 M^{-1}, but in another study Hazum (14) found the affinity for pituitary membranes to be 25×10^9 M^{-1}, which is 33 times larger. We have found (data not shown) that the affinity of our antagonist, (Ac-Δ^3-Pro^1,*p*F-D-Phe^2,D-Trp^3,D-Lys^6)-GnRH, is the same for cultured pituitary cells as for the membrane homogenates. Furthermore, in the work of Hazum (14) the affinity of the agonist [D-Ser-(tBu)^6,des-Gly^{10}-ethylamide]-GnRH as determined by displacement of the labeled agonist is 10 times lower than its value as determined by displacement of the labeled antagonist. In contrast, we have shown that the affinities of a series of GnRH analogues are independent of which radioligand is used.

Hazum (14) also found that the binding of the agonist was reduced by cations to a greater extent than that of the antagonist, and GTP had no differential effects. We have found no differential effects of Na^+, Ca^{2+}, or GTP on the binding of our antagonist. Further support for the conclusion that agonists and antagonists bind similarly to the same set of sites comes from the work of Conn *et al.* (24), in which it was shown that an antagonist can exhibit agonist properties when the antagonist is able to cause receptor microaggregation.

We may conclude from our data that the agonist and antagonist display similar kinetic as well as equilibrium behavior, and that the relative affinities of both agonists and antagonists are independent of which radioligand is used in the radioreceptor assays. This finding provides strong evidence for concluding that the antagonist binds in a competitive manner to the same set of sites available to the agonist. This potent antagonist appears to be a valid radioligand for GnRH pituitary receptor studies, and it should prove useful for studying any possible subtle fundamental differences between agonists and antagonists.

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